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MARTI JETT, Ph.D

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
WALTER REED ARMY INSTITUTE OF RESEARCH
14TH AND DAHLIA STS. NW
BLDG 40, WRAIR
WASHINGTON, DC 20307-5100

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13. ABSTRACT *(Maximum 200 words)*

This study was initiated based on the the fact that there has been little understanding of the role of bioactive lipids in breast cancer, an epocrine gland whose function involves lipid generation. Many of the arachidonate metabolites have been characterized as initiating cascades of other biologically active molecules such as cytokines, activation of kinases and calcium mobilization; these activators of cellular signals have even been shown to alter nuclear receptors which directly interact with DNA to have far-reaching effects on cellular functions. We have found that bioactive lipid pathways were activated by IGF-I in quiescent MCF-7 WT and multidrug-resistant breast cancer cell cultures and have proceeded to examine 6 other cell lines to verify the common pathways involving eicosanoid cascades in response to growth factors. Furthermore, we have found that diverting the above pathways with specific eicosanoid inhibitors stopped proliferation at various points in the cell cycle; thus combinations of these drugs which accumulate cells in different phases of the cell cycle effectively blocked continued cell growth and induced apoptosis. We constructed and developed new drugs, classes of free-radical scavengers, which were effective antiproliferative agents in breast cancer cultures, yet displayed low toxicity to bone marrow cultures and to mice and did not induce multidrug-resistance.

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AIBS 2691

ARACHIDONATE METABOLISM IN BREAST CANCER CULTURES: IDENTIFICATION OF ANTOAGNISTS/ AGONIST FOR POSSIBLE INTERVENTION STRATEGIES.

PRINCIPAL INVESTIGATOR: Marti Jett, Ph.D.

(5) INTRODUCTION:

Arachidonic acid metabolism.

This research proposal was initially prompted by the publication of epidemological data suggesting that people who took low dose aspirin daily for heart disease had a decreased incidence of colon cancer relative to a control group {26}. It was not clear if these individuals had also changed their food habits and life style or if other factors might also have contributed to the observed results. This publication brought out the fact that we had little understanding of the role of arachidonate metabolites in cancer, in general, and in breast cancer, in particular. Many of the arachidonate metabolites have been characterized as initiating cascades of other biologically active molecules such as cytokines, activation of kinases and calcium mobilization; these activators of cellular signals have even been shown to alter nuclear receptors which directly interact with DNA to have far-reaching effects on cellular functions {44}.

Arachidonic acid is released from the Sn-2 position of phospholipids by the family of enzymes phospholipase A2 (PLA2). There are small soluble secreted PLA2's, and PLA2's which are located within the nucleus. The newly released arachidonic acid is, then, the substrate for numerous enzymes including lipoxygenases (LO); a 5-lipoxygenase activating protein (FLAP) is thought to form a complex with 5-LO and anchor it to the inner aspect of the nuclear membrane producing numerous bioactive lipids which initiate the cascades of mediator release. A simplified chart of arachidonic acid metabolism is shown in Fig. 1. The two major classes of enzymes responsible for arachidonic acid metabolism are the cyclo-oxygenases (COX) and lipoxygenases (LO). Cyclo-oxygenases produce the prostanoids and thromboxanes. The best known of these is probably prostaglandin E2, the compound involved in pain and inflammation. Other prostanoids have been implicated in cell proliferation. A multidrug-resistant breast cancer cell line{4,5}, MCF-7 ADR^{10}, has greatly increased levels of prostacyclin H synthetase {28}. Lipoxygenases alter arachidonic acid by first adding a hydroperoxy group (through a free-radical mechanism) at the so named (5-, 12- 15-, etc.) carbon position, and the resulting compounds are named hydroperoxyeicosatetraenoic acids (HPETE). The second generation of compounds are hydroxyeicosatetraenoic acids (HETE's). The lipoxygenases are named for the carbon position which they modify; the 5, 12, and 15 being the most common. The 12- and 15-HETE's have been shown to stimulate cell proliferation in low concentrations {1-3}, however, they become quite toxic at higher concentrations. The 5-lipoxygenase (LO) pathway has

been studied extensively because of its involvement in acute allergy symptoms, shock, adult respiratory distress syndrome{2}. We have identified a 5-LO metabolite, 5-HETE, as a possible growth (co)factor in small cell lung carcinoma and now, as will be described, have implicated it in breast cancer. This compound is also converted to lipoxins, metabolites characterized to stimulate protein kinase C. Lipoxins are abundantly produced in response to growth factors in the tumor cell systems which we are studying. In addition, specifically hydroxylated linoleic acid has been shown to occur upon stimulation of 3T3 cells with EGF {8}. It is unclear if the same lipoxygenase utilize both arachidonic and linoleic acids.

We have used a number of inhibitors of arachidonate metabolism to manipulate the outcome of agonist/antagonist effects. Indomethacin (cyclo-oxygenase inhibitor), eicosatetrayenoic acid, ETYA (a structural analog of arachidonic acid which blocks both cyclo-oxygenase and lipoxygenase pathways), nordiguardaic acid (lipoxygenase inhibitor), and MK591, AA861 (5-lipoxygenase inhibitors). Several of the 5-lipoxygenase inhibitors are in clinical trials for treatment of acute asthma, adult respiratory distress syndrome and arthritis {1-3, 11}. We have used some of these in studies involving toxins, etc. and have demonstrated that these regulators of bioactive lipid generation block proliferation in MCF-7 WT breast cancer cells. Over the course of this study, we have begun to learn that generation of these bioactive lipids is unusually high in breast cancer cultures and blocking a single enzyme involved in these cascades of reactions, while a reasonably effective anti-proliferation strategy, can be improved. Therefore, we have directed many of our studies to examine the use of heteropolyanions which prevent many of the initial electron-transfers and free-radical intermediates essential for utilization of arachidonic acid. In this report, we will show that these drugs have low levels of toxicity toward bone marrow and in mice, are excellent anti-proliferative drugs in cultures of breast cancer cells and the breast cancer cells did not easily develop resistance to these drugs and did not develop multi-drug resistance.

In previous studies from our laboratory, we observed that IGF-I stimulated production of bioactive arachidonate metabolites in NCI-H209 small cell lung carcinoma cultures {43}. In that study we found that (a) 5-LO products were overproduced (b) in quiescent cultures, 5-HETE stimulated cell proliferation, (c) inhibitors of 5-LO metabolism blocked proliferation, (d) the 5-LO enzymes were over-expressed in response to IGF-I. In this report we will present preliminary evidence that similar pathways are crucially involved in proliferation in cultures of MCF-7 WT breast cancer cells.

Study of AA861 inhibition of proliferation in the breast cancer cell line, MCF-7 WT showed that cell proliferation {6} was completely blocked by the 5-lipoxygenase, AA861 (collaborative studies with R.L. Fine, Duke/Va Medical Center, Durham, NC). Synchronized cells held by thymidine block in G_0, when released from the block, took 2-3 hr to develop cell surface markers typical of the G_S phase, and arachidonate analysis from 15min to 5 hr showed that 5-HETE production increased rapidly upon washing out the block and continued to accumulate for about 90 minutes. As 5-HETE

production fell, an elevation was seen in the 5-HPETE and 15-HETE metabolites. Interestingly, asynchronous control cultures displayed increased 5-HETE production upon fluid change, although the time of production was delayed and the quantity produced was markedly less. We have proposed that 5-HETE has characteristics of a growth (co)factor.

(6) BODY

MATERIALS:

Materials: Phenol red-free IMEM (improved minimal essential medium; BioFluids, Walkersville, MD); Complete culture fluid containing 7% fetal bovine serum, 1% MEM vitamins and 1% antibiotics; all from BioFluids, Walkersville, MD; bioactive lipid standards and many specific inhibitors were purchased from BioMol Inc., Plymouth Meeting, PA.

Cell cultures: MCF-7 WT cells were grown in phenol red-free IMEM to avoid problems with estrogen-like activities of phenol red. Cells were subcultured twice per week to prevent irreversible clumping. Cultures were discontinued after 15 subcultures and new cultures brought up from frozen stock. Cultures were tested weekly for estrogen receptor status. We have used these cultured cells for approximately 8 years.

METHODS:

1. Establish quiescence in cultures of MCF-7 cells. In the previous report, we showed that quiescence by serum depletion for 2 days produced cells which could respond to stimuli. This basic study showed that i) cells did not detach from the matrix, ii) cells had a decreased proliferation rate after 2 days culture in limited nutrients, and iii) cells recovered from the limited nutrient period and show proliferation both by thymidine incorporation and by microscopically determining the number of viable cells. The procedure can be summarized as follows:

a) Day 1: Plate cells at a density which will permit them to remain in culture 1 week (10,000 /2 sq. cm. Plate in complete medium containing 7% fetal bovine serum and other usual additives. (Serum is necessary for the cells to attach to the plastic dishes).

b) Day 3: Remove the fluid, gently wash the cultures with saline. Replace with serum-free medium containing NO additives.

c) Day 4: Add agonist/antagonists to study generation of bioactive lipids.

2. Description of proliferation assays to assess inhibition of cellular growth. We evaluated 4 different types of proliferation assays last year in order to adopt labor saving techniques and have a confirmatory assay to assess inhibitors which may interfere with the assay, per se, rather than with proliferation. The use of a fluorescent ligand to DNA was the ideal assay since (a) cells did not need to be trypsinized and harvested, therefore reducing labor and inconsistencies (b) it was unnecessary to wash cells free of serum and proteins (c) samples had to be frozen to release intracellular DNA, therefore, the proliferation assay could be run at the convenience of the researcher rather than at a the end of a specific time period for incubation with drugs.

3. Screening of inhibitors of the lipoxygenases, cyclo-oxygenases, and other bioactive lipids to identify those which have antiproliferative activity at acceptable concentrations. The methods used in these studies are described in the previous section.

4. Synthesis of heteropoly anion free-radical scavengers: Syntheses of high oxidation state manganese-substituted heteropolyanions. We first isolated and characterized Mn^{IV} substituted Keggin polyanions ($[XW_{11}\ Mn^{IV}\ O_{40}\ ]^{n-}$, X = Si, B, and Zn) using X-ray diffraction, Extended X-ray absorption fine structure method, magnetic susceptibility, electrochemistry, and routine spectroscopic methods {35}. Di-manganese substituted γ-Keggin polytungstosilicates, γ-$[SiW_{10}\ Mn_2\ O_{40}\ ]^{m-}$, were synthesized{22-25, 29-34}. Oxidation of tetra-manganese substituted polyaions, $[P_2\ W_{18}\ Mn_4\ O_{68}\ \bullet 2H_2\ O]^{10-}$ game two mixed valent compounds were synthesized and characterized {28} as previously described {35-38}. Oxidation of alkylene with iodosobenzene (PhIO) was examined using above mono-, di-, and tetra manganese-substituted polyanions in the presence and absence of air. The oxidized products were identified by GC-Mass and comparison the retention time with authentic compounds

5. Determination of inhibition of proliferation in breast cancer cell cultures by inhibitors of bioactive lipid generation or heteropoly anion free-radical scavengers: The techniques described previously have been used for determination of proliferation in breast cancer cell cultures. Heteropolyanions have been shown to have antiproliferative activities {39-41}

6. Determination of toxicity to cultures of human bone marrow cells by inhibitors of bioactive lipid generation or heteropoly anion free-radical scavengers. Studies to determine if there is a reasonable "therapeutic window" of differential toxicity to normal bone marrow cultures *Vs* breast cancer cells:

#1. Separation of light-density bone marrow cells. Human bone marrow was obtained from previously screened donors. Marrow specimen were diluted 1:1 (vol./vol.) in sterile calcium and magnesium-Dulbecco's phosphate-buffered saline (DPBS). Twenty mL of diluted marrow cells were layered over an equal volume of ficol gradient and centrifuged at 400g for 30 min. at room temperature. The buoyant nucleated marrow cells were collected from gradient interfaces and washed twice by centrifugation with DPBS. The cells were suspended in Lone-Term Culture Medium (LTCM).

#2. Stroma colony assay was performed in LTCM using methods adopted from Dr. Vincent La Russa and others {42, 7}. Above $2x10^5$ light-density cells were plated in 4 well plates with 5 mL LTCM in the absence or in the presence of heteropolyanions or inhibitors. The cells were incubated at 37C, 5% CO_2, and 100% humidity for two or four weeks. The medium with heteropolyanions or inhibitors were changed every week. The stroma colonies were fixed and staining after two and four weeks. The stained stroma colonies were washed and dried in the air. The total number of colonies were counted.

#3. Colony assay for hematopoietic progenitor cells was carried out in methylcellulose cultures (4). Light-density cells (10^5 cells/plate) were plated in 35-mm-diameter gridded tissue culture plates with 1 mL methylcellulose mediun in the absence or in the presence of heteropolyanions or inhibitors. The methylcellulose medium contains 1.1% methylcellulose

in IMDM, 30% (vol./vol) FBS, 2.5 U/mL of human recombinant erythropoietin, and 10% (vol./vol) giant-cell tumor conditioned media. The cultures were incubated at 37C, 5% CO_2 , and 100% humidity. The hematopoitic colonies were counted using an inverted phase microscope at 14-21 days. CFU-GM, BFU-E, and CUF-Mix were recognized using standard criteria of colonal morphology.

#4. Human bone marrow stroma cells were cultured for three weeks in LTCM with weekly changing medium. The stroma were unattached and plated in 35-mm diameter gridded tissue culture plates in LTCM. After 24 h, the mediun was changed to heteropolyanions in LTCM and incubated for a week. The heteropolyanion medium was removed and the cells washed with 1X PBS buffer solution. The untreated human bone marrow light-density cells in methylcellulose (the same as described in Experiment #2 without GCT) were plated on the top of the treated stroma cells. After the incubation, colony formation was determined using an inverted phase microscope at 14-21 days. CFU-GM, BFU-E, and CFU-Mix were recognized using standard criteria of colonal morphology. IC50 values in normal cells Vs tumor cells are being compared so that we can select ca. 3 HPA's.

7. **Arachidonate metabolism**: HPLC separation, identification and quantitation of ca. 30 arachidonate metabolites in a single run was devised by our laboratory {27}. We have established a pattern for determination of the kinetics of formation of even transient arachidonate metabolites in cell cultures +/- agonists or antagonists. The method can be adapted for the separation, and identification of radioactive metabolites from cell cultures, or for separating, identifiying and quantitating minuscule amounts (fMol & pMol) in samples from animals or tissues.

8. **Agonist stimulated arachidonate metabolism**: IGF-I stimulates proliferation quiescent MCF-7 cultures {43}. The objective of this proposal is to identify bioactive lipids which are being generated in response to growth factor stimulation in the presence and absence of specific arachidonate inhibitors or heteropolyanion treated MCF-7 cells. The MCF-7 cell cultures (an adherent cell line) will be plated in 4-well cluster plates and 24 hr later will be incubated overnight arachidonic acid (approximately 10 uCi/well). We have found this culture pattern to produce metabolites with sufficient radioactivity. The culture fluid will be removed, a saline wash performed, and buffer added (buffer designed for optimal phospholipase A2 activity). Control and agonist/antagonists will be added and the reaction stopped (acidified) at selected time periods from 15 sec through 2 hr (about 12 different time points for each series (control, agonist, etc.). We usually limit an experiment to approximately 50 wells. The cells will be scraped from the wells, briefly sonicated to insure cell disruption (some arachidonate metabolites may remain intracellular), and centrifuged at 20,000 x g to pellet all insoluble material. The internal standard is then added to the supernatant solution, the pH adjusted, arachidonate metabolites extracted and the samples prepared and run on reverse phase using a C-18 column for HPLC analysis. Platelet activating factor fraction will be separated away from arachidonate metabolites at the extraction step and will be assayed. Meanwhile, the pelleted material will be extracted by Bligh-Dyer procedure and the extracts applied to thin layer plates to run in solvent systems designed to resolve phospholipids or diglycerides/fatty acids. The latter techniques give interesting

information when comparing phospholipids present at the beginning of the experiment (samples under 2-3 minutes) with those present at the end of the experiment (>2 hr).

9. Toxicity testing in mice: HPA-Sm was administered to Balb C, 6-8 week old mice using a 12 gauge mouse lavage tube for oral administration or was administered subcutaneously on the back. Plasma levels of drug were determined using atomic absorption spectrometry.

RESULTS AND DISCUSSION:

1. Classes of inhibitors/drugs which prevent formation and/or utilization of bioactive lipids, were found to be equally effective in blocking proliferation in MCF-7 breast cancer cells of (A) wild-type (WT), or (B) multidrug-resistant (ADR). Graphs not displayed in this report, show that inhibitors of bioactive lipids effectively block proliferation at low IC50, in 6 additional breast cancer lines besides the two described in detail in this report (abstract enclosed describing this work Appendices 1).

a) Five-lipoxygenase (5-LO) inhibitors (Figure 2, A & B). MK591 and MK886 are each inhibitors of the 5-LO activating protein (FLAP). The site of their inhibition is shown in Figure 1 (pathway B). These two inhibitors were found to be equally effective in blocking proliferation in WT or ADR breast cancer cultures; in both cultures, the 5-LO inhibitors showed IC50 of 8-16 uM. Nordiguaretic acid (NDGA) is a non-specific lipoxygenase inhibitor and the site of its inhibition is shown in Fig. 1, pathway A. The non-specific LO inhibitor, NDGA was not as effective with MCF-7 ADR cells as with WT cells.

b) AA861 (5-LO) inhibitor exerts it primary actions at the site depicted in Fig. 1, pathway C, although it also affects pathway B, to a lesser extent. MCF-7 WT cultures were reasonably sensitive to this drug (Figure 3 A); we now do not believe that it is not a drug of great promise, however. We have previously shown that cyclo-oxygenase (COX) inhibitors (Fig.3, pathway D) such as aspirin or Indomethacin stimulated rather than blocking proliferation in breast cancer cells. We examined dual LO/COX inhibitors such as Curcumin and found that it was more effective with ADR than with WT cells (Figure 3, A & B). However, we found that it displayed toxicity to bone marrow cultures (described subsequently). Other drugs which also inhibited lipoxygenase production via a different route, such as the Cytochrome P450 blocker, ketoconazole, was very effective in stopping proliferation in ADR cultures (Figure 3B).

c) Heteropolyanions (HPA), free-radical scavengers (Figure 4, A & B). The simplified arachidonate metabolic pathway chart (Fig 1) depicts intermediate products along the pathway to formation of some very potent bioactive lipids. In each step, free-radical formation is necessary for enzymatic conversion to the next product, therefore, we examined an array of HPAs to evaluate their ability to block proliferation (and generation of bioactive lipids) in breast cancer cells. These drugs were effective at IC50 concentrations from about 0.5-3 uM. These concentrations show little if any toxicity to bone marrow cell cultures. Structures of representative HPA's are shown in Figure 5.

d) Platelet activating factor (PAF) receptor antagonists (Figure 6). No difference was observed between wild type and multidrug-resistant MCF-7 breast cancer cells in regards to inhibition of proliferation by PAF receptor antagonists (Fig 6), or calcium mobilization disruption (Figure 7).

d) Protein kinase C (PKC) inhibitors (Figure, 8 A & B). Two inhibitors of protein kinase C activation show similar IC50 for proliferation. Interestingly, the sphingosine IC_{50} for ADR cells was half of that seen for WT cells.

2. The corollary of the above study: will breast cancer cells easily develop resistance (or become multidrug-resistant) to these drugs or inhibitors which block the formation of bioactive lipids? Part of the logic in examining these drugs is that they associate with cellular membranes rather than accumulating in the cytoplasm, therefore, they should be sequestered and unaffected by the multidrug-resistance efflux pump (P-170 glycoprotein).

a) Table 1. It is not easy for cultures of MCF-7 breast cancer cells to be able to survive in increase concentrations of heteropolyanions (columns 1 and 2). Note the relatively higher increases in concentration of Adriamycin, by comparison (column 3). After 3 months of slowly but steadily increasing concentrations, the breast cancer cells could survive in increased concentrations of HPA or Adriamycin.

c) Figure 9, A & B. The cellular marker for multidrug-resistance (P-170 glycoprotein) was identified in MCF-7 cells exposed to steadily increasing concentrations of Adriamycin but not in cultures treated with heteropolyanion drugs. (A) Western blot using either antisera C219 or 4E3 detected P170 in the ADR cultures. (B) Fluorescence microscopy using a fluorescent derivative of the antisera designated 4E3, which recognizes a portion of the extracellular domain of P-170 glycoprotein, showed that P170 was abundant on breast cancer cells made resistant to Adriamycin, but was not detected on the parent WT breast cancer cells or cells made resistant (to some degree) to HPA drugs.

3. Synchronized cells were examined for their kinetic pattern of advancing through the cell cycle in the presence of various drugs. The importance of this study is that it will be a mechanism to identify which drugs are inducing apoptosis or are arresting cells in specific phases of the cell cycle. This will provide important information regarding the stage of cellular development and action of various bioactive lipids, and the optimal combinations of drugs to maximize blocking cellular proliferation.

a) An example of the results is shown in Figure 10 to illustrate cells in various phases of the cell cycle. This is a graph of control MCF-7 ADR cells 16 h after synchronization. Note that at this time period, the cells were cycling from G2/M-phase back to Go/G1.

b) To further illustrate the cycling, kinetic studies were performed from 2-24 h (Figure 11).
The series of graphs shown in column 1, illustrates the synchrony of control, untreated cells in Go/G1 at 2h, their movement into S-phase (4-10 h), the accumulation in G2/M-phase by 12 h, and the return to Go/G1 starting at 16 h and reaching near synchrony, again, by 20 h. Graphs in column 2, cells treated with curcumin, show that the drug blocks the cells from advancing out of Go/G1. Graphs in column 3, cells treated with NDGA, show that the cells accumulate in

G2/M-phase, rather than Go/G1. Many drugs exert their effects in Go/G1, therefore, identification of NDGA as a drug which prevents the advance from G2.M-phase, is most useful for combination therapy.

4. Toxicity testing in human bone marrow (a) stroma cells or (b) hemapoetic colony forming cells suggests that heteropoly anion drugs were more toxic to tumor than to normal cells; an example of these studies is shown (Figure 11 A & B). We have now found that SmP-HPA could be increased to about 20 uM in cultures of bone marrow cells. Also shown are toxicity levels with a number of drugs which block bioactive lipid production (Figure 11B and Table 2).

5. In preparation for evaluating these drugs in a breast cancer model in mice, experiments were carried out to determine the doses and time schedule for administration of the heteropolyanion drugs. Therefore, mice were injected with doses of SmP-HPA from 0.15 to 20 mg/mouse. 20 mg/mouse was toxic after about 8-16 h; organs were examined upon necropsy, but no abnormality was observed. Cardiotoxicity is a common side effect of antiproliferative drugs, and that is suspected in this case. Administration of 7 mg x 2 one week apart was also toxic from about 48-96 h. A dose of 1.65 ug/mouse x 4, weekly showed no toxicity other than the loss of hair. Necropsy did not reveal any gross lesions in any of the organs. Comparisons were made administration of 0.15 ug x 14 (2.1 mg total) weekly injections with 0.7 ug x 3 (2.1 mg total) weekly injections. In both cases necropsy did not reveal toxicity other than hair loss. Route of administration was compared and plasma concentrations determined at various time points after administration of drug. Table 3 shows that subcutaneous administration provided a steady release of drug appearance in plasma; plasma concentrations were still elevated at 24 h. In comparison, oral administration of the drug showed a lower plasma concentration, however it appeared to be rising at 24 h. At 48 h, levels were approximately the same as at 24 h.

WORK ACCOMPLISHED COMPARED TO STATEMENT OF THE WORK (in appendices):

1. During these first two years of this research project, we have completed Year 1, parts 1, 2 and 3; Year 2, parts 1, 2; Year 3, part 1 and 2. Because of problems with vendor supplied CO2 for our incubators (see special problems, listed below), we had difficulty completing Year 2, Parts 3 and 4. We are well on our way in that study, however. It was necessary to substitute 2 objectives (1 and 2) from Year 3, since they could be carried out independently of cell cultures and, therefore, were not affected by the problems with our incubators.

FUTURE DIRECTIONS AND GOALS:

1. The most urgent objective is to complete the arachidonate metabolite generation studies in the presence and absence of inhibitors, especially the heteropolyanions. Generation of arachidonate metabolites in response to IGF-I (in quiescent breast cancer cell cultures) is the major thrust of this proposal. In this study, the kinetics of arachidonate metabolite

generation will be observed in control and IGF-I stimulated cultures. We will follow the generation and utilization of up to 30 metabolites in an effort to understand the pathways responsible for generation of bioactive lipid intermediates.

2. Alterations is generation of bioactive lipids in the presence of identified inhibitors will be used to confirm our hypothesis that preventing the formation of certain of these compounds will disrupt growth of breast cancer cells which are so dependent on these signaling cascades. These studies will be carried out in quiescent cultures, preincubated for 30 min with the appropriate inhibitor(s), in control and IGF-I stimulated cultures.

3. Determine the involvement of kinases and their relationship to arachidonate metabolism in IGF-I and control breast cancer cell cultures.

PROBLEMS ENCOUNTERED:

In late January, we lost all cell lines which were in culture. It was perplexing since we could not detect contamination of any sort. Furthermore, all new cultures, even those plated by ATCC, would not survive in our incubators (it was not immediately obvious that the problem was connected to our incubators). We replaced incubator filters, hoses, etc., without success. We examined all new culture fluids and plasticware which we had received. The mystery was solved in late-April when the supplier of gases disclosed that they had inadvertently sold us CO2 contaminated with cyanide (see notification of contamination in addendum). This had a devastating effect on our progress this year in terms of performing research which was logical to do in sequence. Instead, we skipped to studies intended for FY97, since they could be carried out independently of the cell cultures.

ACCOMPLISHMENTS:

1. International Patent Rights submitted: USE OF LIPOXYGENASE INHIBITORS AS ANTICANCER THERAPEUTIC AND INTERVENTION AGENTS. James Mulshine and Marti Jett. Mar 14, 1996. Pending.

MANUSCRIPT:

1. A.W. Panandiker, T. Boyle, **Marti Jett**, Sam Sorof. Mitogenesis by target protein of carcinogens: Selective depressions of arachidonic acid metabolites by fatty acid binding protein and peroxisome proliferator. We expect to submit this in January to J. Nat Cancer Inst.

ABSTRACTS:

1. Y. Wang, X. Zhang, J. Weitz and M. Jett (1996). Heteropolyanions do not Induce P-Glycoprotein associated with multidrug Resistance in Human Breast Cancer Cells. FASEB Journal 10:A1145.

2. XiaoYan Zhang, R. Tran, J. Weitz and **Marti Jett** (1996). Manipulation of arachidonate metabolism in cultures of MCF-7 breast cancer cells. Proceedings of the Am Assn for Cancer Research, Proceedings.

(7) CONCLUSIONS

a) We have shown that bioactive lipid pathways were activated by IGF-I in quiescent MCF-7 WT and multidrug-resistant breast cancer cell cultures and have proceeded to examine 6 other cell lines to verify the common pathways involving eicosanoid cascades in response to growth factors.
b) Furthermore, we have found that diverting the above pathways with specific eicosanoid inhibitors stopped proliferation at various points in the cell cycle; thus combinations of these drugs which accumulate cells in different phases of the cell cycle effectively blocked continued cell growth and induced apoptosis.
c) We constructed and developed new drugs, classes of free-radical scavengers, which were effective antiproliferative agents in breast cancer cultures, yet displayed low toxicity to bone marrow cultures and to mice and did not induce multidrug-resistance.
d) We determined that IGF-I activated the MAP kinase signaling cascade and other downstream events and are moving forward in studying the signaling sequences activated by growth factors..

(8) LITERATURE REFERENCES

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(9) APPENDICES

STATEMENT OF THE WORK

YEAR ONE

I. Characterize MCF-7 WT cell culture proliferation and/or inhibition in the presence of specific inhibitors of bioactive lipid generation including, arachidonate metabolism, such as aspirin/indomethacin, 5-lipoxygenase inhibitors, 15-lipoxygenase inhibitors or specific product inhibitors of 15-lipoxygenase metabolites.

II. Establish the conditions for making MCF-7 cells quiescent. Determine that addition of growth factors or serum will induce proliferation in the quiescent cultures.

III. Characterize MCF-7 WT cells with regard to proliferation in response to IGF-I and other appropriate growth factors.

YEAR TWO

I. Characterize the conditions for synchronizing cells in the G_0, resting cycle and then determine the time required for the cultures to advance to G_S. This will require the use of flow cytometry techniques to determine DNA with characteristics of specific cell cycle stages. Evaluate promising drugs/ inhibitors in this system to determine the phase in which they block proliferation. Use this as a basis for selecting drugs which have the potential to be most effective when used in combination.

II. Determine if breast cancer cells easily become resistant to selected promising inhibitors determined above and determine if they induce multidrug-resistance. These drugs which block bioactive lipid synthesis/ expression are designed to associate with cellular membranes rather than the cytosol and therefore we project that they will not easily be pumped out of multidrug-resistant cells; compare the effect of these drugs in blocking proliferation in multidrug-resistant cells as compared with wild-type cells.

III. Characterize arachidonate metabolism in MCF-7 WT cells kinetically in samples +/- IGF-I and establish the effect of antagonists such as glucagon, on IGF-I stimulated cells.

IV. Determine the arachidonate metabolites formed upon treating the cultures with aspirin, 5-lipoxygenase inhibitors and other appropriate inhibitors, depending on the outcome of the proliferation experiments.

YEAR THREE

I. With the knowledge gained from the previous two years of proliferation studies, apply information concerning growth factors, antagonists, and/or inhibitors to other breast cancer cell lines to see if common themes emerge. Test for synergy among various metabolic inhibitors.

II. Specific inhibitors identified as being unusually effective in blocking proliferation in breast cancer cultures will be evaluated for toxicity to normal human bone marrow (a) stroma cells and (b) to hemapoetic colony forming cells. Depending on the outcome of these studies, perform *in vitro* studies in mice to determine non-toxic dosing regimens.

II. Establish the relationship of JAK/STAT or RAS/RAF signaling cascades and their relationship with the generation of bioactive lipid metabolites; examine lipoxygenases for phosphorylation in response to IGF-I in quiescent cells. Determining the contribution of signaling kinases in quiescent cells in response to IGF-I and/or other growth factors.

III. Closely examine the arachidonic acid metabolites and/or other biologically active metabolites such as cytokines, if indicated, which appear during the period of time between resting cells (G_0) and G_S.

IV. Examine other breast cancer cell lines +/- growth factor(s), and inhibitors identified in the previous years to see if stimulation/inhibition of identified arachidonate metabolites can be confirmed as a general finding in breast cancer.

YEAR FOUR

I. Identify possible correlations between growth factor stimulation of specific arachidonate metabolites which are found to be common in breast cancer, identify the most effective inhibitor(s) and their possible combinations for potential intervention strategies for further investigations and possible clinical trials for treatment of breast cancer.

II. Depending on the outcome of studies using specific inhibitors of arachidonate metabolism *in vitro*, examine animal models of breast cancer to see if the selected inhibitor(s) are effective *in vivo* in arresting breast cancer tumor development.

III. Summarize accumulated data

Abstract

Arachidonic acid metabolism has an important function in promoting the growth of cancer cells. Previously, we demonstrated that the lipoxygenase pathway of arachidonic acid metabolism is important in the growth regulation of lung cancer, we now report a similar role for this pathway in breast cancer. In this study, the six breast cancer cell lines analysed express mRNA for 5-lipoxygenase, and FLAP. In contrast, mRNA for 12, and 15-lipoxygenase were not uniformly expressed (3/6), and (5/6), respectively. We also show evidence that clinical specimens of malignant and normal breast tissue express mRNA for 5-lipoxygenase. Two exogenously added autocrine growth factors IGF-I and TF, induced the biosynthesis of the 5-lipoxygenase metabolite 5-HETE. Using a proliferation growth assay, our results demonstrate that 5-HETE can directly stimulate growth (>25% over control at 0.01 ug/ml) *in vitro*. Furthermore, inhibition of lipoxygenase metabolism by selective antagonists at 1-10 uM concentrations resulted in significant, reproducible growth reduction of 20-90% *in vitro* of the tumor cell lines tested. Inhibitors of the 5-lipoxygenase activating protein were most potent. In contrast, a cyclooxygenase inhibitor at 10-100 uM had no significant effect. Breast cancer cells exposed to lipoxygenase inhibitors demonstrate increased frequency of apoptosis, which is consistent with the mechanism for the anti-proliferative effect of the inhibitors being mediated via upregulation of apoptotic growth control. These results suggest that inhibitors of the lipoxygenase pathway may provide a new pharmacological method to reduce the growth of breast cancer cells.

ABBREVIATIONS

SCIENTIFIC TERMS:

Lipoxygenase (LO); phospholipase A2 (PLA2); 5-lipoxygenase activating protein (FLAP); cyclo-oxygenase (COX); hydroperoxyeicosatetraenoic acids (HPETE); hydroxyeicosatetraenoic acids (HETE); epidermal growth factor (EGF); Insulin-like growth factor (IGF); Improved minimal essential medium (IMEM);

DRUGS/INHIBITORS:

MK 591, A gift from Merck-Frosst. A 5-Lipoxygenase activating protein inhibitor. The designation/name was supplied by the manufacturer.

MK 886, A gift from Merck-Frosst. A 5-Lipoxygenase activating protein inhibitor. The designation/name was supplied by the manufacturer.

Eicosatetrayenoic acid (ETYA)

AA861 (manufacturer's designated name), a purchased inhibitor of 5-lipoxygenases

Chelerythrine (CHEL)

Nordihydroguaiaretic Acid (NDGA)

Heteropolyanions (HPA), see figure 5; Sm= Samarium in a HPA; Na= Sodium in an HPA, etc.

Bone Marrow Assay system:

Colony-Forming Units (CFU); Erythroid (E), Granulocyte, Macrophage (GM); Granulocyte, Erythroid, Macrophage, Megakaryocyte (GEMM); All of the above (MIX); Burst-Forming Units (BFU).

LEGENDS TO FIGURES

Figure 1. A schematic diagram showing some of the major bioactive lipid metabolites of arachidonic acid. Pathway A indicates non-specific lipoxygenases; B, 5-lipoxygenases, C, peptidoleukotrienes; and D, cyclo-oxygenases.

Figure 2. Proliferation Assay: Five-lipoxygenase (5-LO) inhibitors with wild type (WT) cells (A) or multidrug-resistant cells (B). Cells were plated in 96 well cluster plates as described in Methods; the drugs were added the next day (after the cells had attached to the culture dish). MK591 and MK886 are each inhibitors of the 5-LO activating protein (FLAP); the site of inhibition is shown in Fig. 1, pathway B. Nordiguaretic acid (NDGA) is a non-specific lipoxygenase inhibitor and the site of its inhibition is shown in Fig. 1, pathway A.

Figure 3. Proliferation Assay: AA861 (5-LO) inhibitor exerts it primary actions at the site depicted in Fig. 1, pathway C, although it also affects pathway B, to a lesser extent. MCF-7 WT cultures (A) and multidrug-resistant cultures (B) were also treated with dual LO/COX inhibitors, such as Curcumin (Figure 1, Pathways A and D). Another drug also inhibited lipoxygenase production via a different route, Cytochrome P450 blocker, ketoconazole.

Figure 4. Proliferation Assay: Heteropolyanions (HPA), free-radical scavengers (Figure 4, A & B) block intermediate product generation in arachidonate metabolism (depicted in Fig 1). The conditions for assay are described in the legend to Fig. 2.

Figure 5. Structures of representative Heteropolyanions.

Figure 6. Proliferation Assay: Platelet activating factor (PAF) receptor antagonists was evaluated for blocking proliferation in WT (A) or multidrug-resistant (B) MCF-7 breast cancer cells. PAF is a by-product of the released arachidonic acid (see Fig. 1). The conditions for assay are described in the legend to Fig. 2.

Figure 7. Proliferation Assay: Calcium release from intracellular organelles stimulation (blocks Ca^{++}-ATPase) by thapsagargin. Enzymes involved in the metabolism of eicosanoids are calcium-dependent. The conditions for assay are described in the legend to Fig. 2.

Figure 8. Proliferation Assay: Protein kinase C (PKC) inhibitors was evaluated for blocking proliferation in WT (A) or multidrug-resistant (B) MCF-7 breast cancer cells; inhibitors of PKC also block eicosanoid metabolism. The conditions for assay are described in the legend to Fig. 2.

Figure 9. The cellular marker for multidrug-resistance (P-170 glycoprotein) was identified in MCF-7 cells exposed to steadily increasing concentrations of Adriamycin but not in cultures treated with heteropolyanion drugs. (A) Western blot using either antisera C219 or 4E3 detected P170 in the ADR cultures. (B) Fluorescence microscopy using a fluorescent derivative of the antisera designated 4E3, which recognizes a portion of the extracellular domain of P-170 glycoprotein. Frames in 9B: MCF-7 WT breast cancer cells (a), MCF-7 ADR^{10} multidrug-resistant cells (b), MCF-7 cells treated with HPA SM500 (C) or HPA Na500 (D) for 3 months to attempt to develop drug resistance.

Figure 10. Synchronized cells were examined for their kinetic pattern of advancing through the cell cycle in the presence of various drugs to identify which drugs are inducing apoptosis or are arresting cells in specific phases of the cell cycle. An example of the results is shown in Figure 10 to illustrate cells in various phases of the cell cycle. This is a graph of control MCF-7 ADR cells 16 h after synchronization.

Figure 11. To further illustrate the cycling, kinetic studies were performed from 2-24 h. The series of graphs shown in column 1. Graphs in column 2, show cells treated with curcumin, and graphs in column 3 show cells treated with NDGA.

Figure 12. Toxicity testing in human bone marrow (a) stroma cells or (b) hemapoetic colony forming cells to determine relative toxicity of bioactive lipid inhibitors and heteropoly anion drugs to normal Vs breast cancer cells.

TABLE I

ATTEMPT TO FORCE DRUG RESISTANCE TO HETEROPOLY ANIONS AS COMPARED TO ADRIAMYCIN.

NUMBER OF DAYS OF TREATMENT*	$[SmP_5W_{30}O_{110}]$	$[NaP_5W_{30}O_{110}]$	ADRIAMYCIN
DAY 1	0.10 μm	0.10 μm	10 μm
DAY 4	0.10 μm	0.10 μm	20 μm
DAY 7	0.13 μm	0.13 μm	40 μm
DAY 10	0.13 μm	0.13 μm	80 μm
DAY 14	0.20 μm	0.20 μm	160 μm

*Cells were incubated with each of these drugs, separately, at concentrations indicated. Column 1 indicates the number of days (time course) required for cell recovery/survival before proceeding to an increased concentration of the drug.

TABLE I I

Hemapoetic colony distributions

Inhibitors - Concentration	BFU-E	CFU-E	CFU-GM	CFU-MIX	CFU-GEMM
control	30	43	55	13	4
MK591-16	24	24	70	14	3
MK591-40	37	26	63	12	3
MK591-70	10	16	24	0	0
NDGA-16	26	25	61	12	4
NDGA-40	4	4	18	1	0
NDGA-70	0	0	0	0	0
PCA-13	29	24	93	12	3
PCA-38	29	15	71	4	2
CHEL-10	26	28	83	21	4
CHEL-20	24	30	40	2	2
CHEL-40	11	18	0	0	0
MK-886-8	33	25	91	12	4
MK886-30	28	21	56	8	2

Bone marrow cultures were established to evaluate toxicity induced by the potential drugs/inhibitors tested in the breast cancer cultures. Bone marrow toxicity is one of the major problems associated with chemotherapy. The drugs were tested at selected concentrations (μM), shown to the right of the drug designation. Figure 1 illustrates the various pathways involved for inhibition of eicosanoid metabolism and the following drugs block at the designated part of the cascade. MK-591 and MK-886 are inhibitors of FLAP (Figure 1, pathway B); nordiguaiaretic acid (NDGA) is a non-specific lipoxygenase inhibitor (Pathway A); PCA is an inhibitor of platelet activating factor production (Figure 1); Chelerythrine (CHEL) blocks protein kinase C activation and indirectly alters eicosanoid metabolism. The concentrations chosen were based on IC_{90} with MCF-7 WT cells.

TABLE III

SmP-HPA CONCENTRATION IN MOUSE PLASMA: ORAL vs SUBCUTANEOUS ADMINISTRATION[a]

TIME AFTER ADMIN-ISTRATION OF DRUG	CONCENTRATION OF HPA IN PLASMA SUBCUTANEOUS ADMINISTRATION	CONCENTRATION OF HPA IN PLASMA: ORAL ADMINISTRATION
2 h	3.5 ug/mL	0.24 ug/mL
4 h	3.1 ug/mL	0.15 ug/mL
6 h	4.5 ug/mL	0.17 ug/mL
24 h	4.4 ug/mL	0.83 ug/mL

[a] The dose of SmP-HPA administered was 1.65 mg/mouse. At the time periods indicated, the mice were euthanized in an atmosphere of CO_2 , cardiac puncture performed to obtain the blood, and the plasma separated by centrifugation at 1500 x g for 20 min at 4°C. The samples were digested with nitric acid and then neutralized with NaOH, and the samples subjected to atomic absorption spectral analysis.

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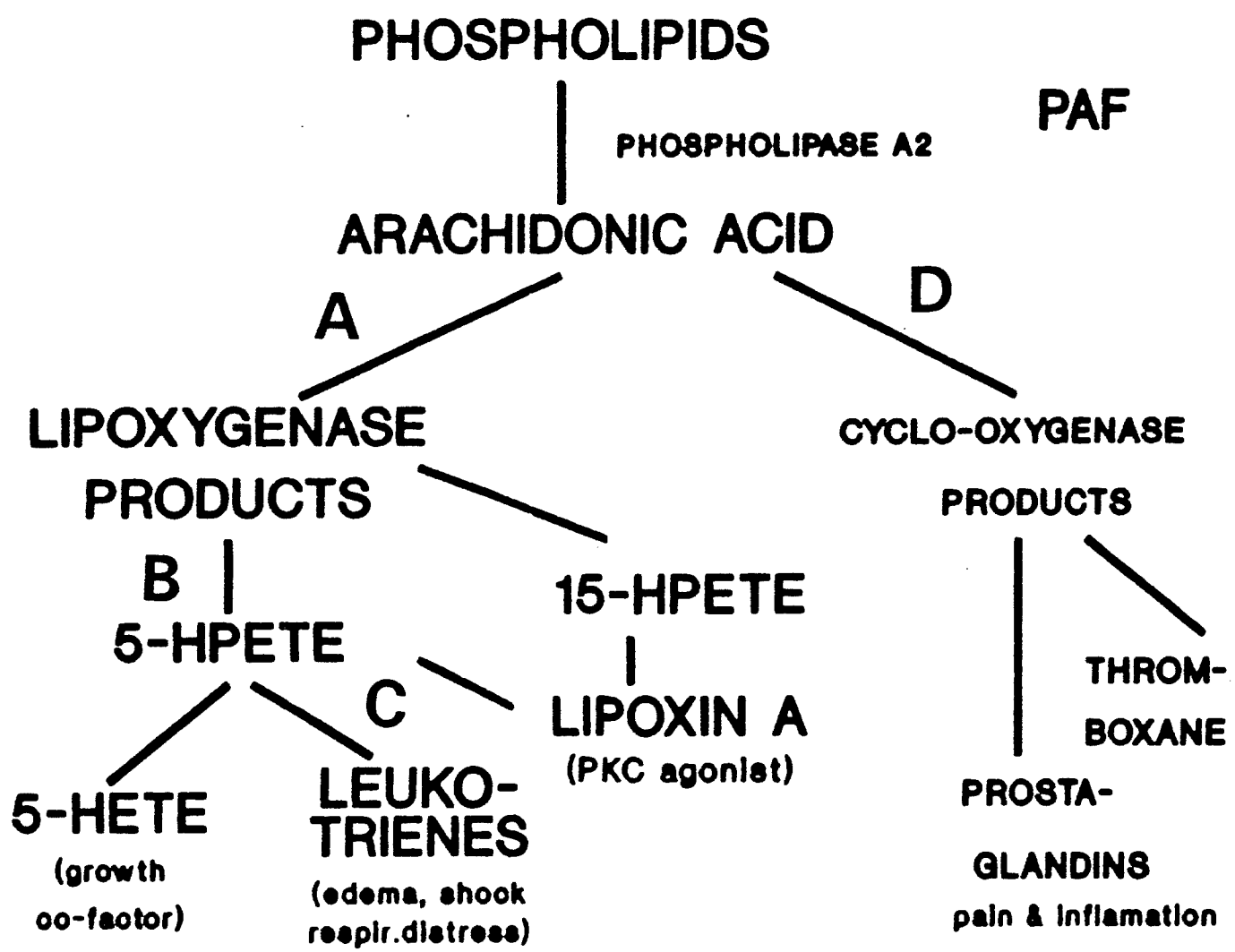

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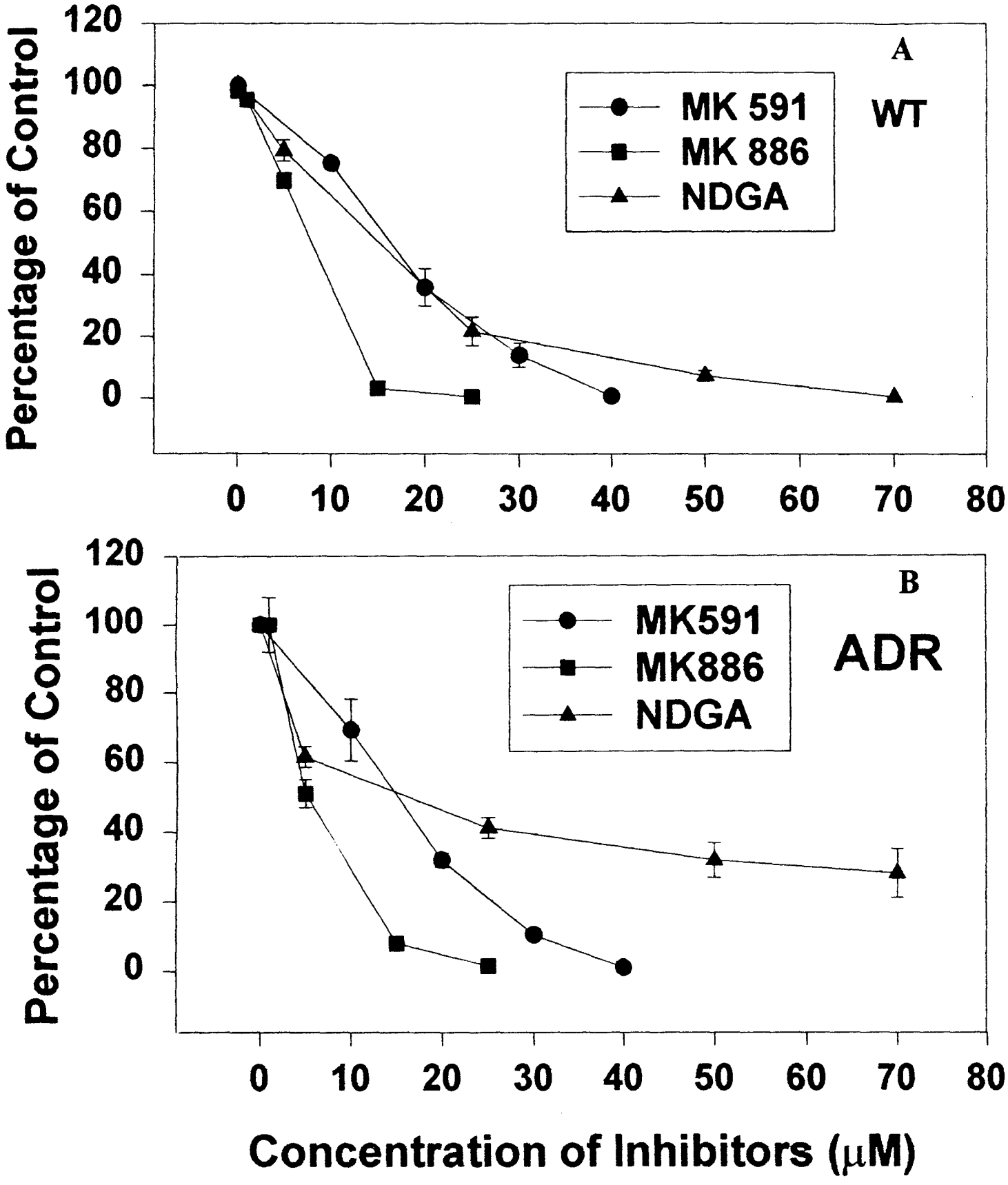
CELL PROLIFERATION



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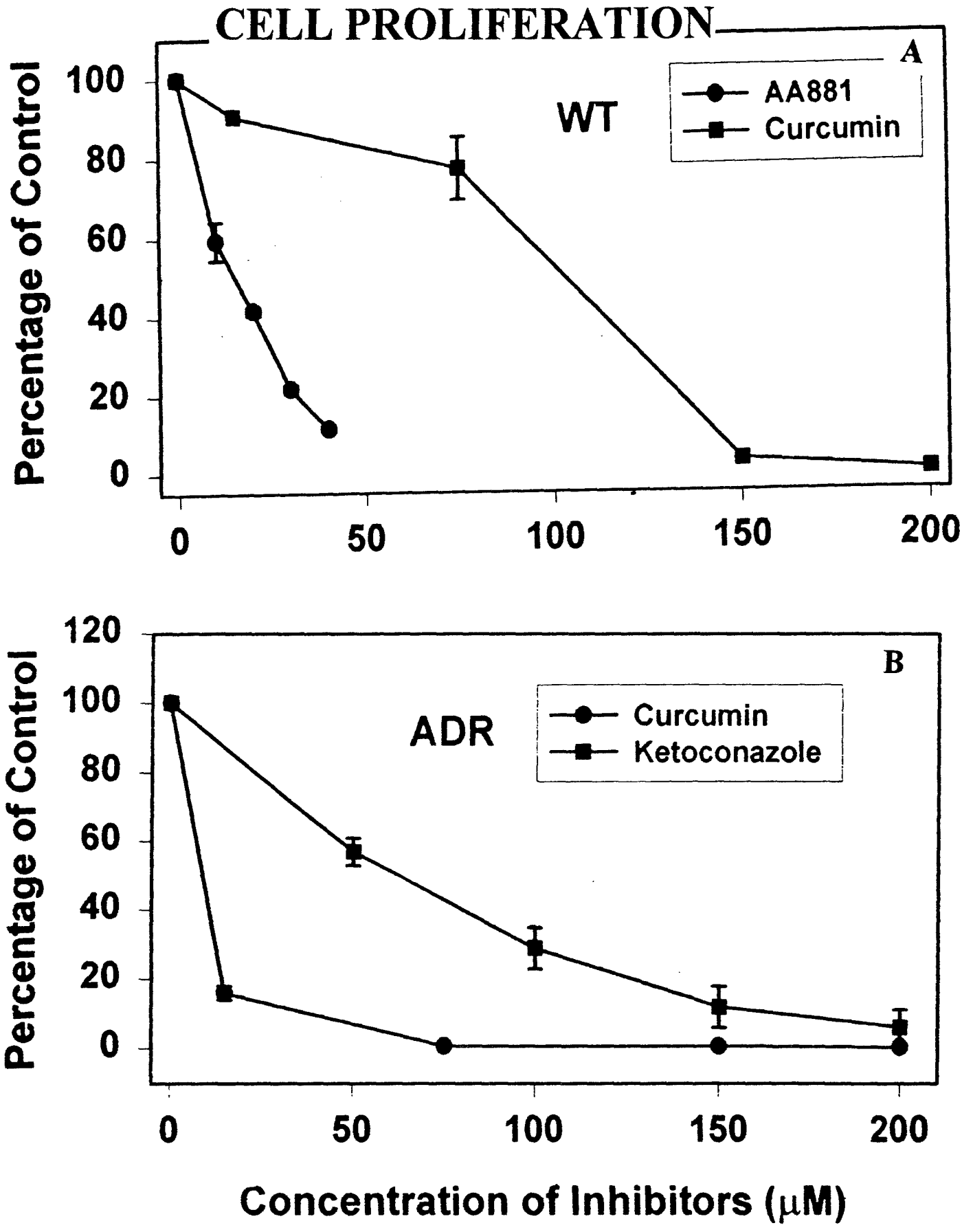

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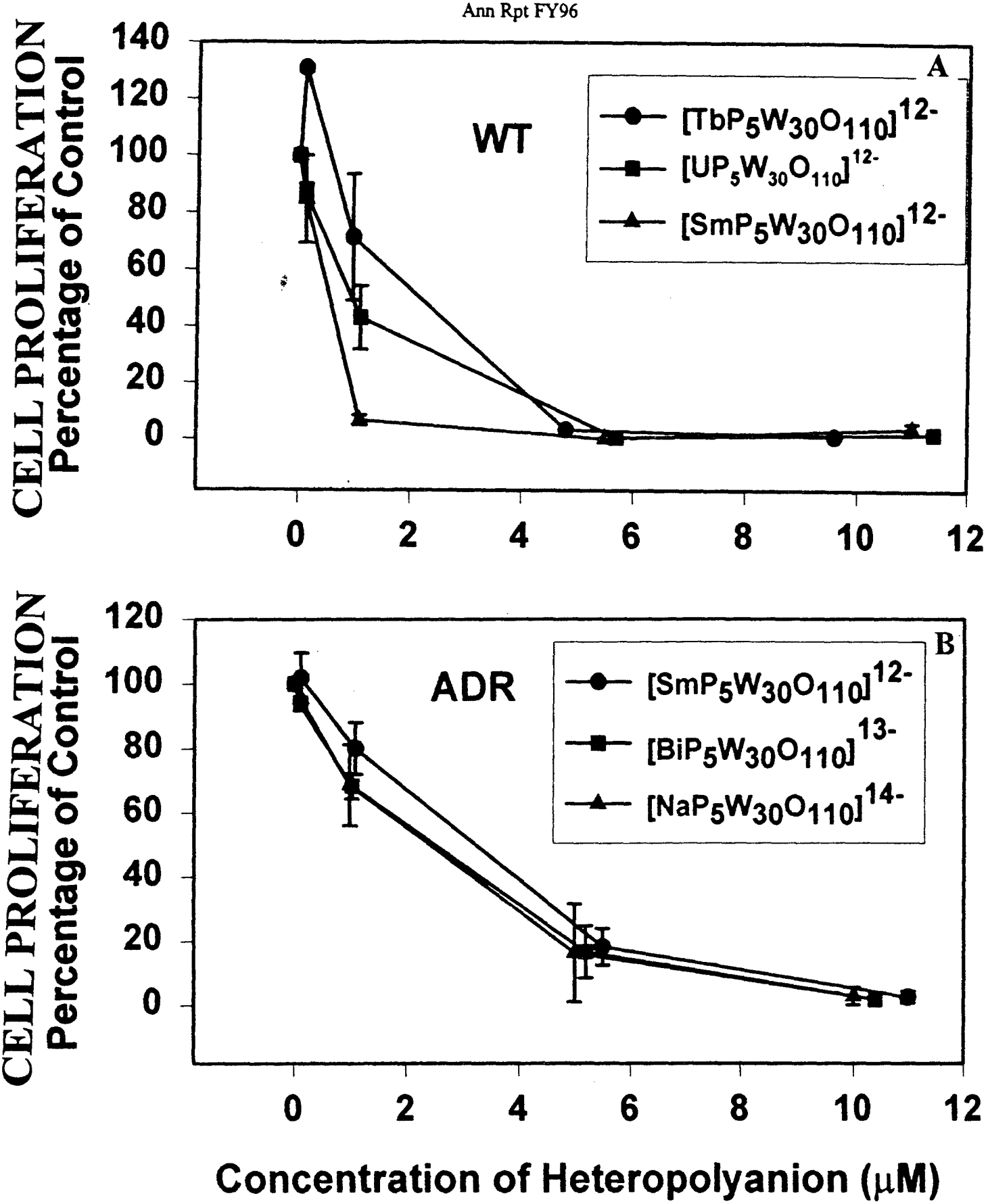

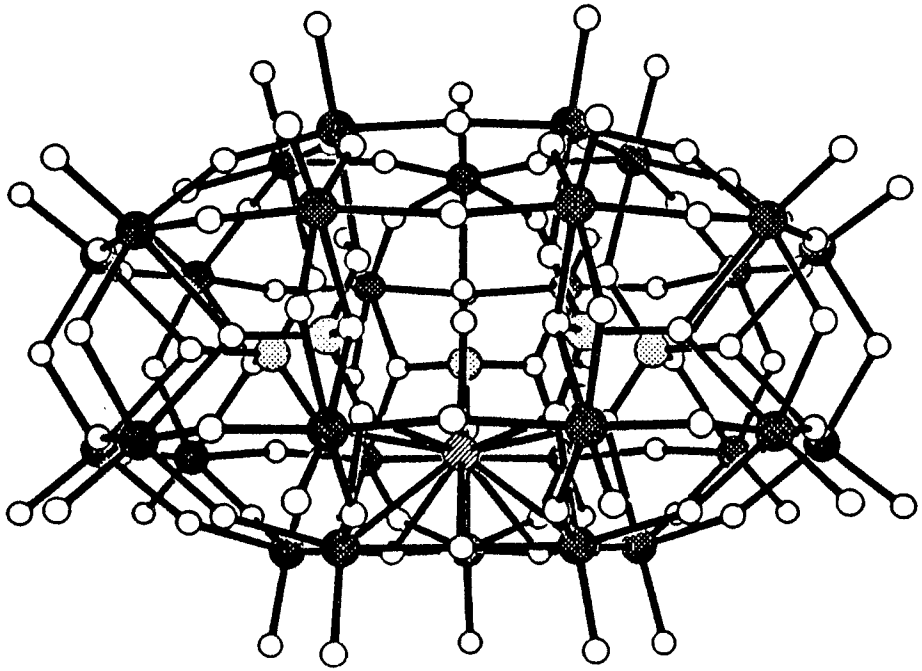

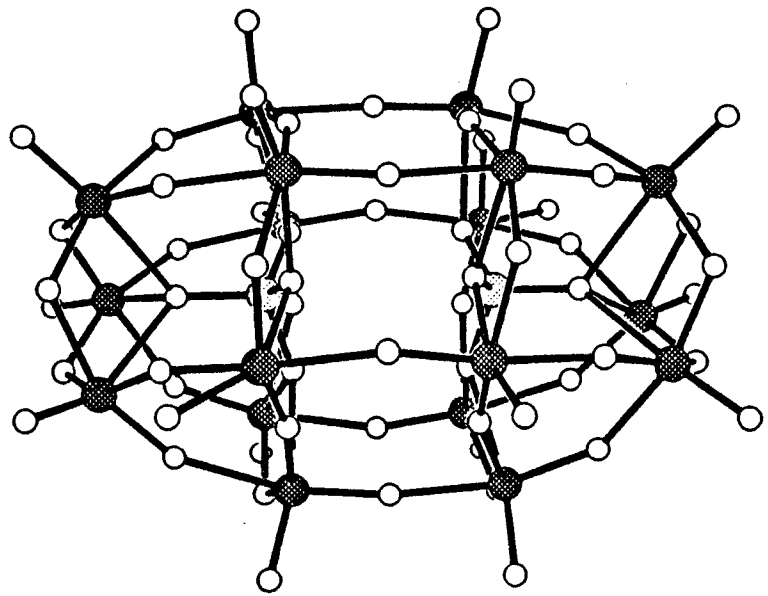

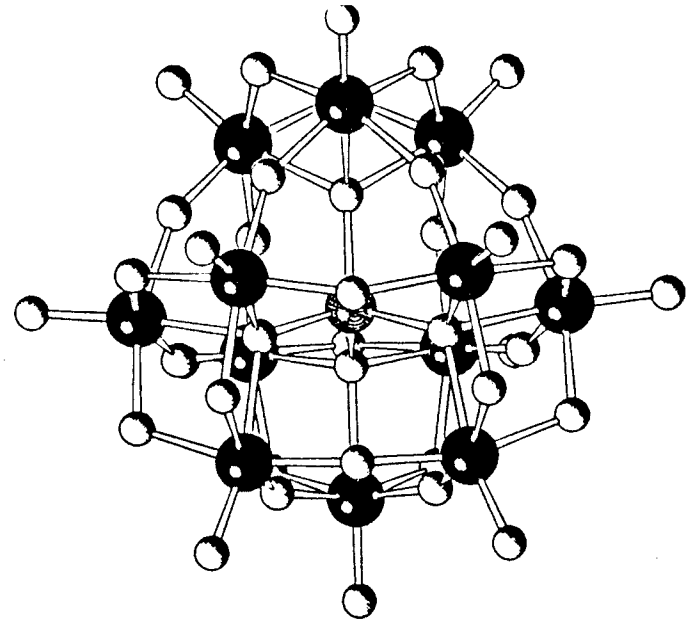

Figure 5 The structure of $[X(PO_4)_5W_{30}O_{90}]^{n-}$, X = Na^+, Ca^{2+}, Bi^{2+}, Ce^{3+}, Sm^{3+}, Eu^{3+}, Gd^{3+}, Tb^{3+}, Dy^{3+}, Ho^{3+}, Er^{3+}, Tm^{3+}, Yb^{3+}, Lu^{3+}, or U^{3+}.

X: ● ; W: ● ; P: ● ; O: ○

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Figure 6
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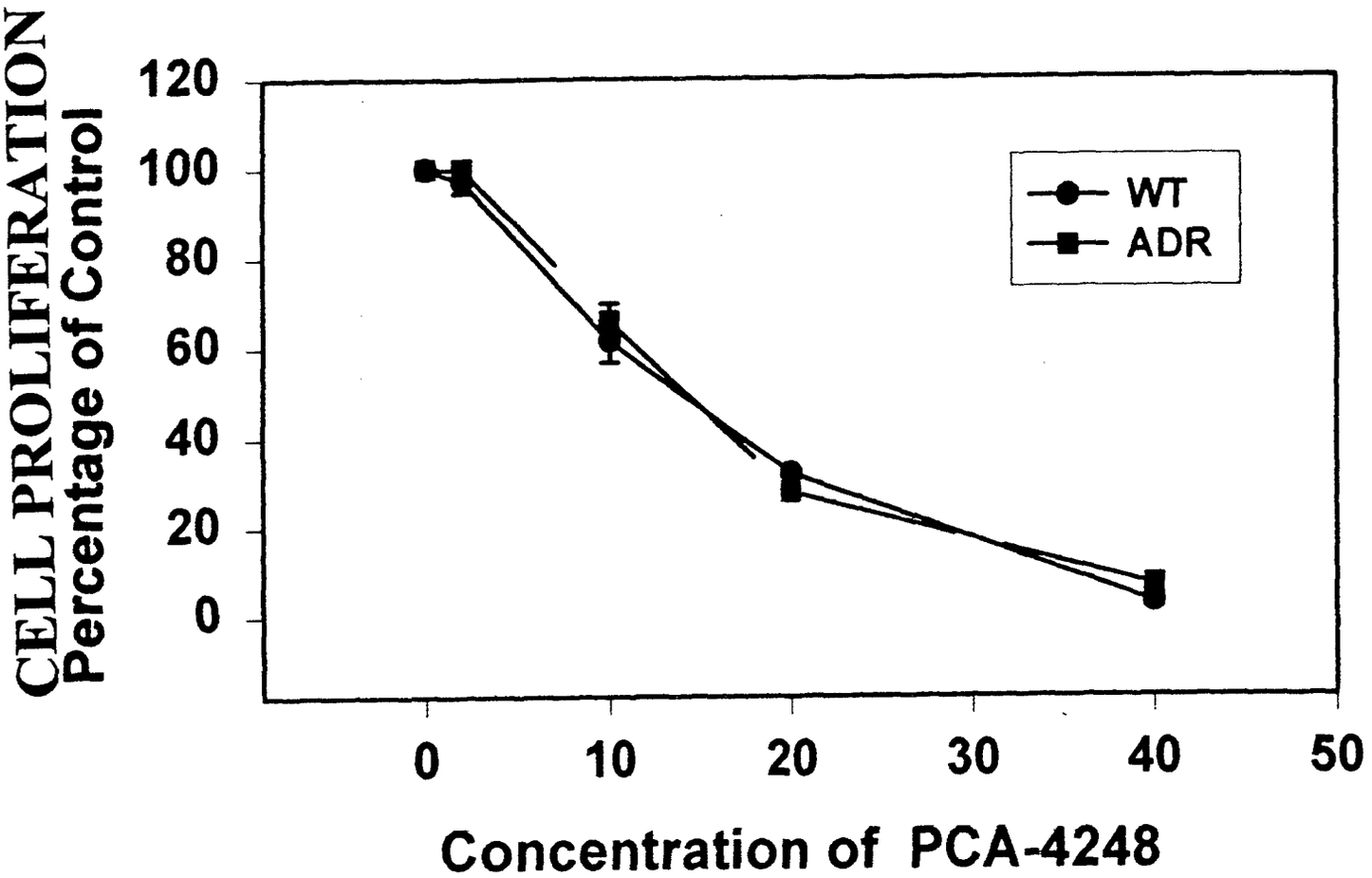

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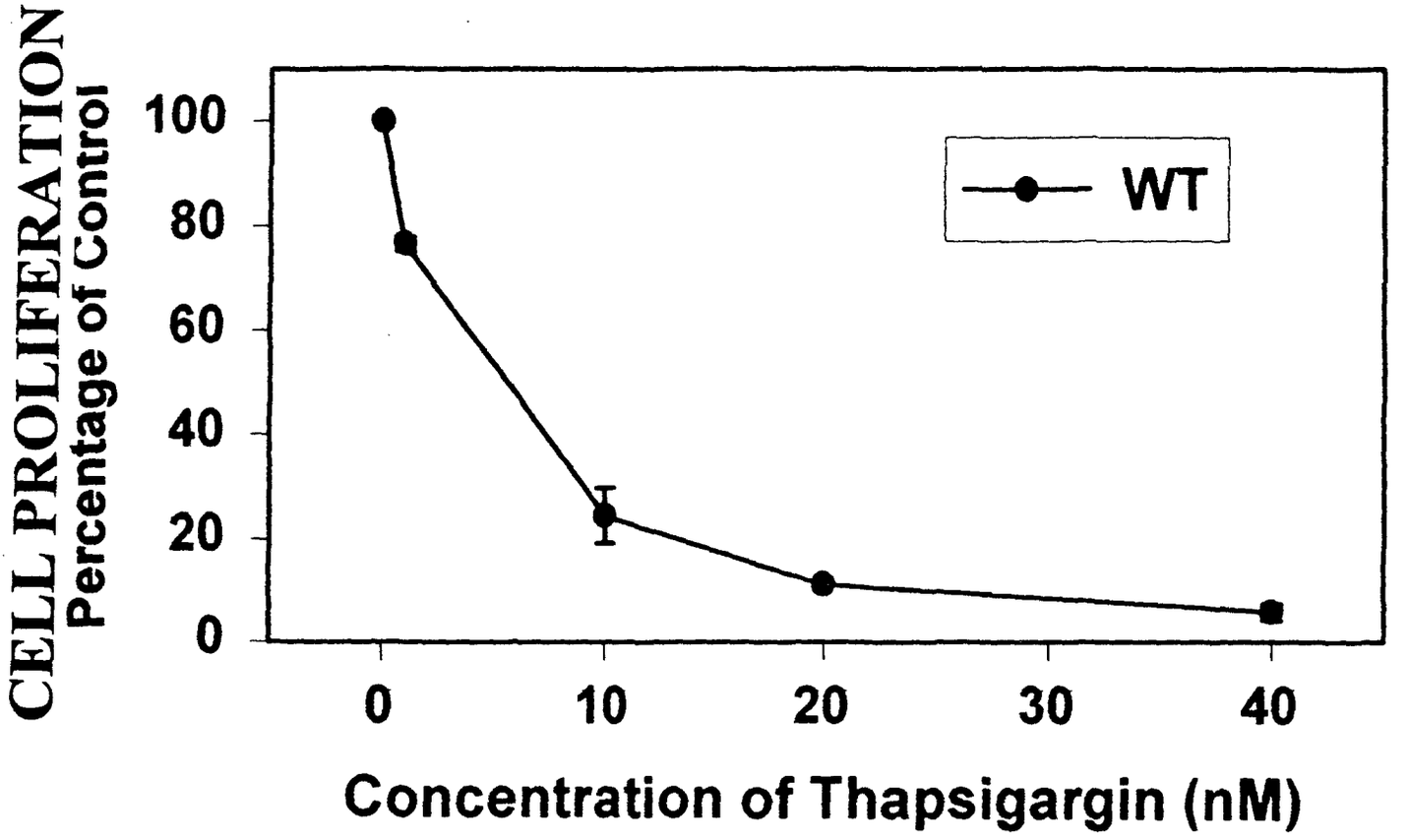

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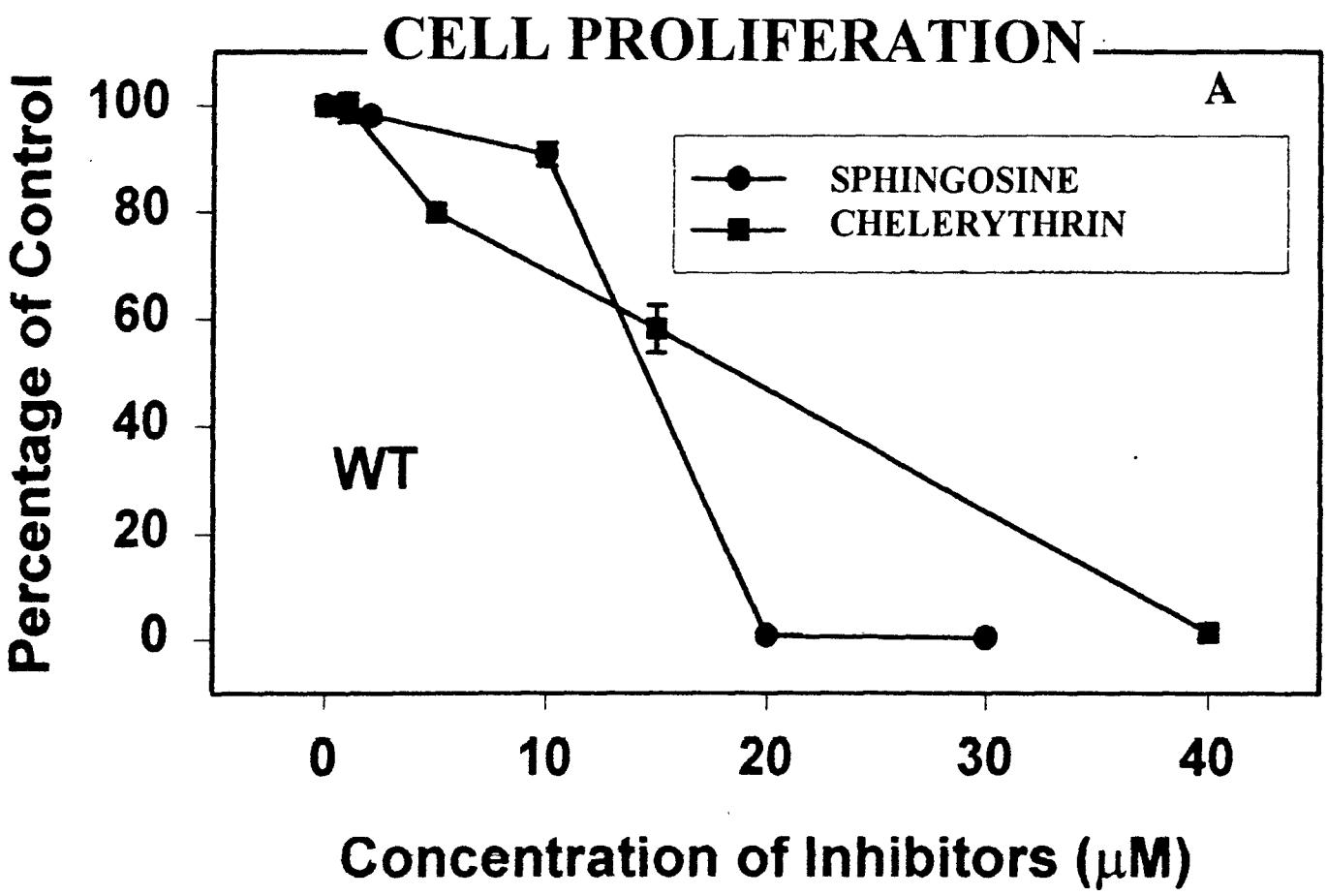

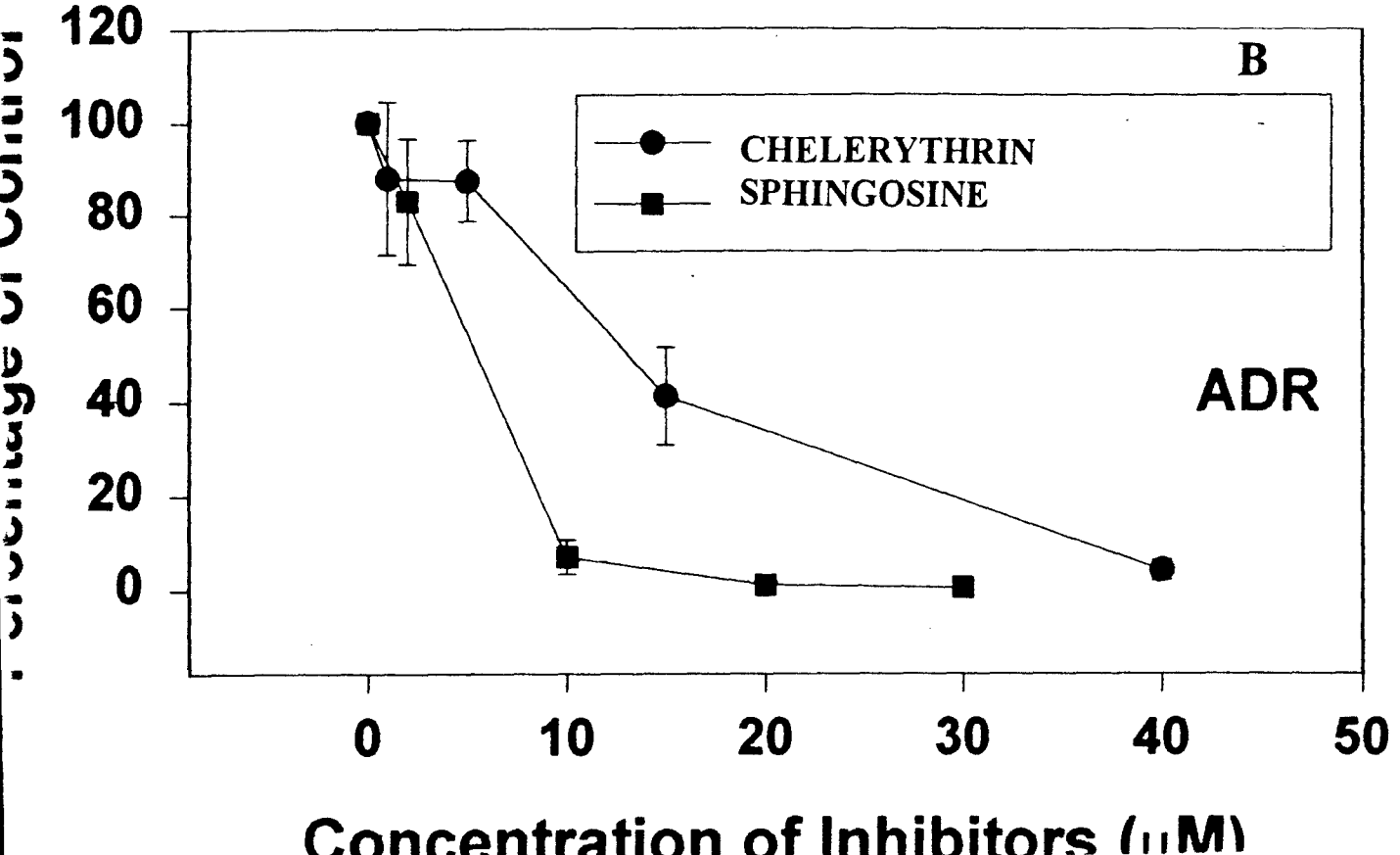

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Figure 9A
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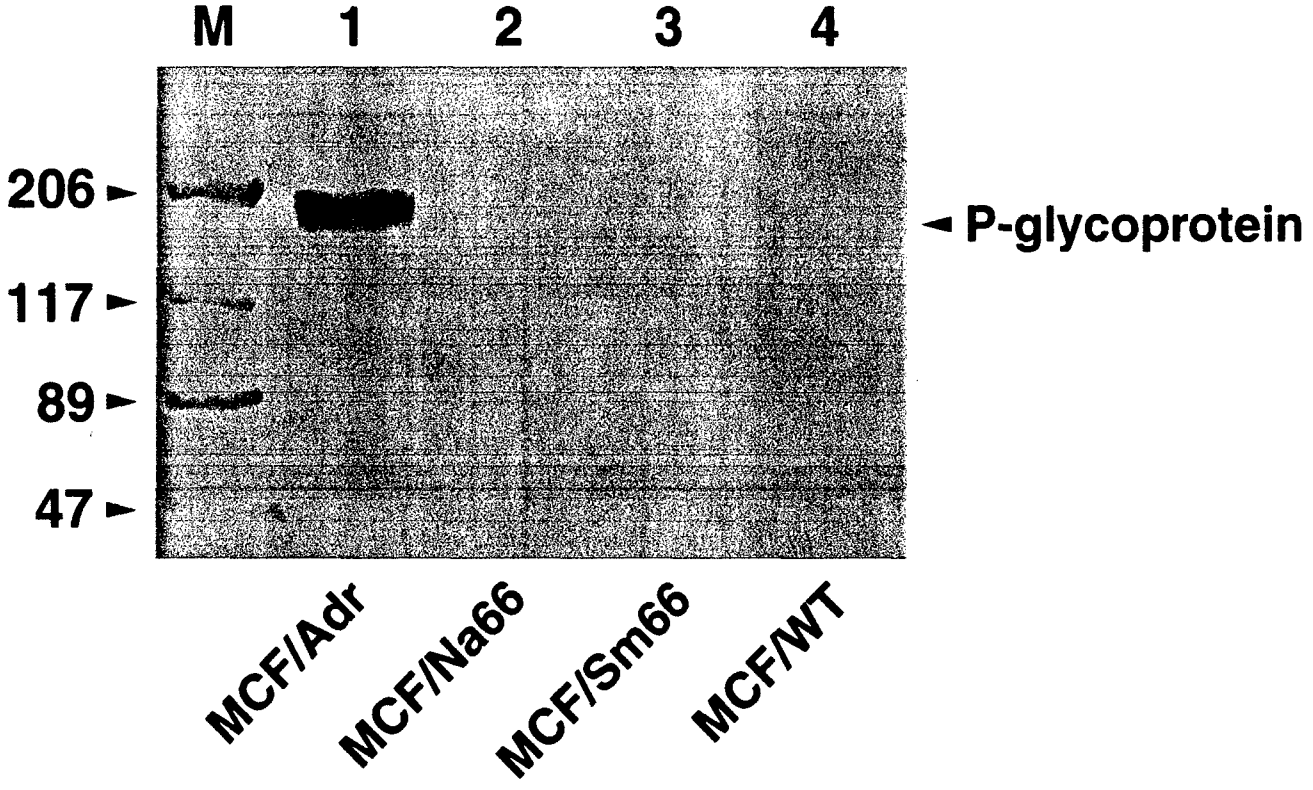

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Figure 9 B
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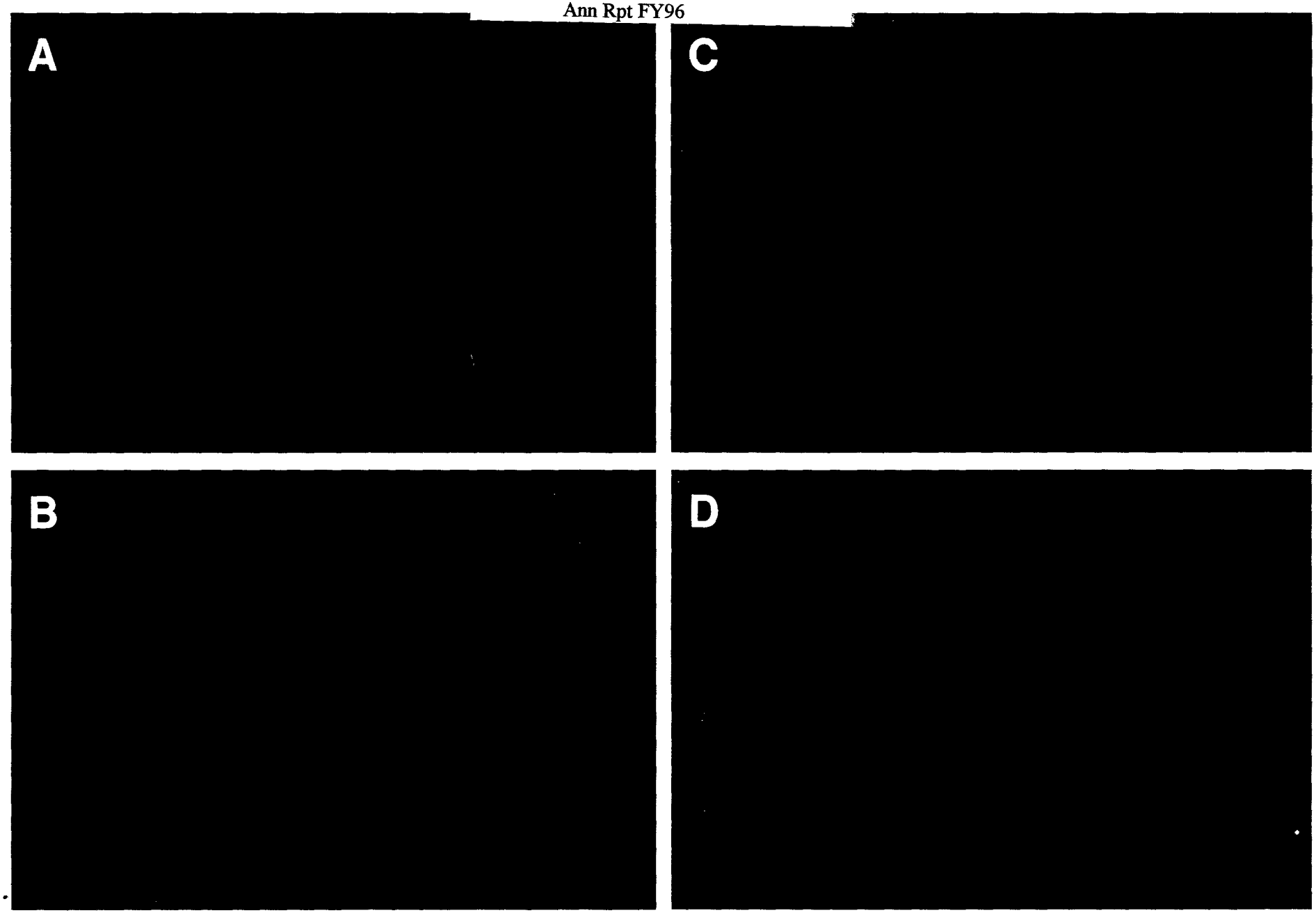

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Figure 10
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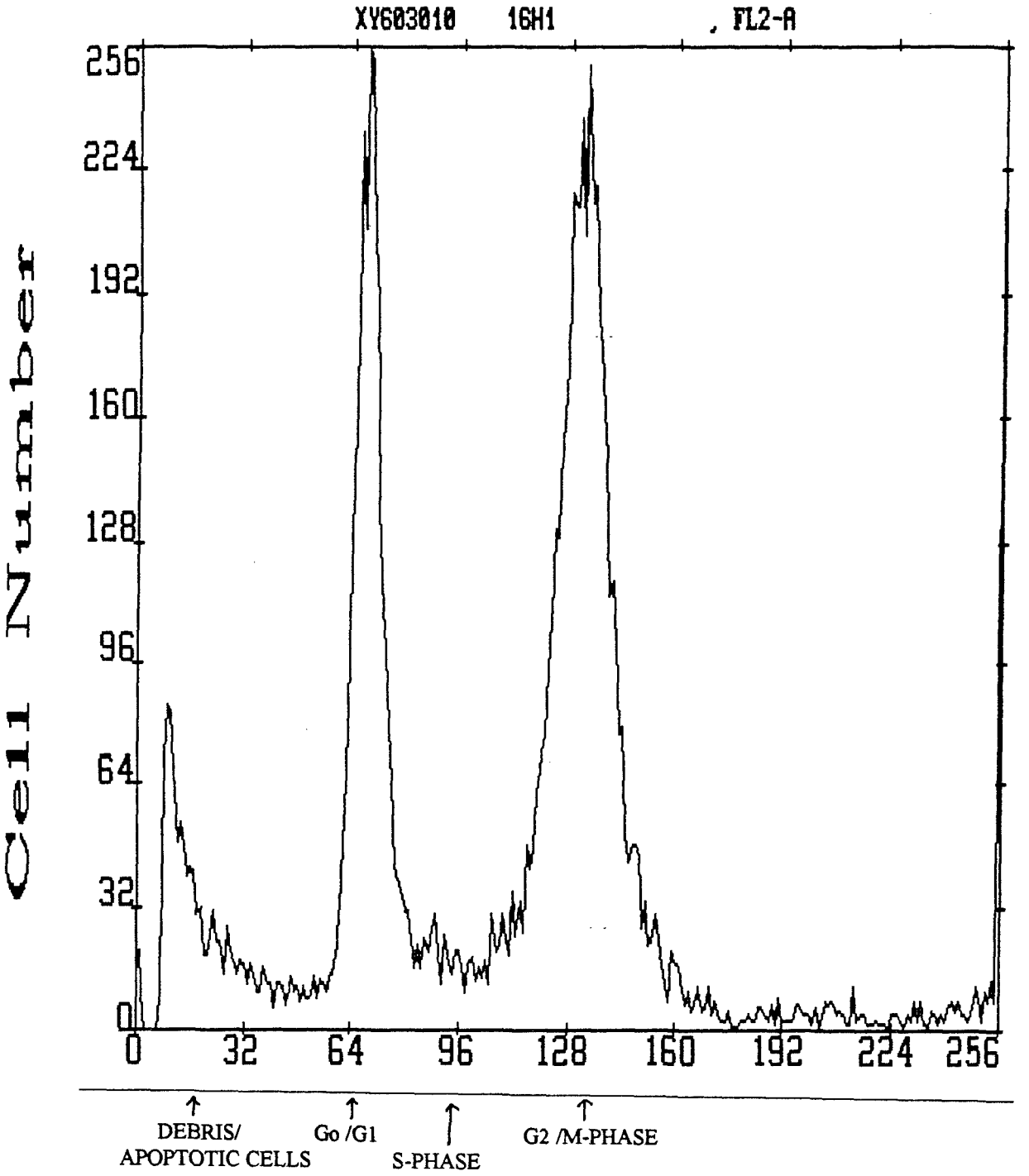
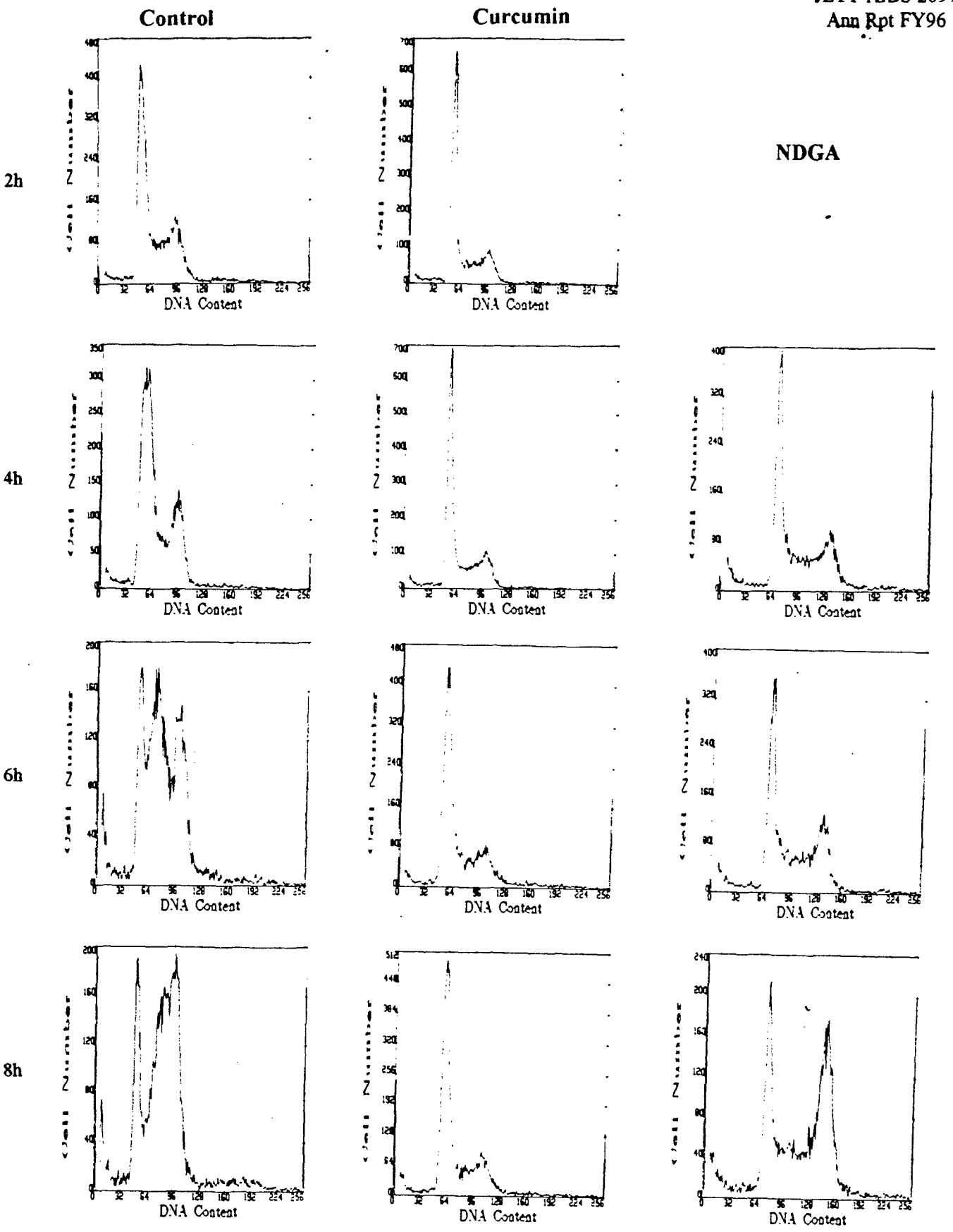


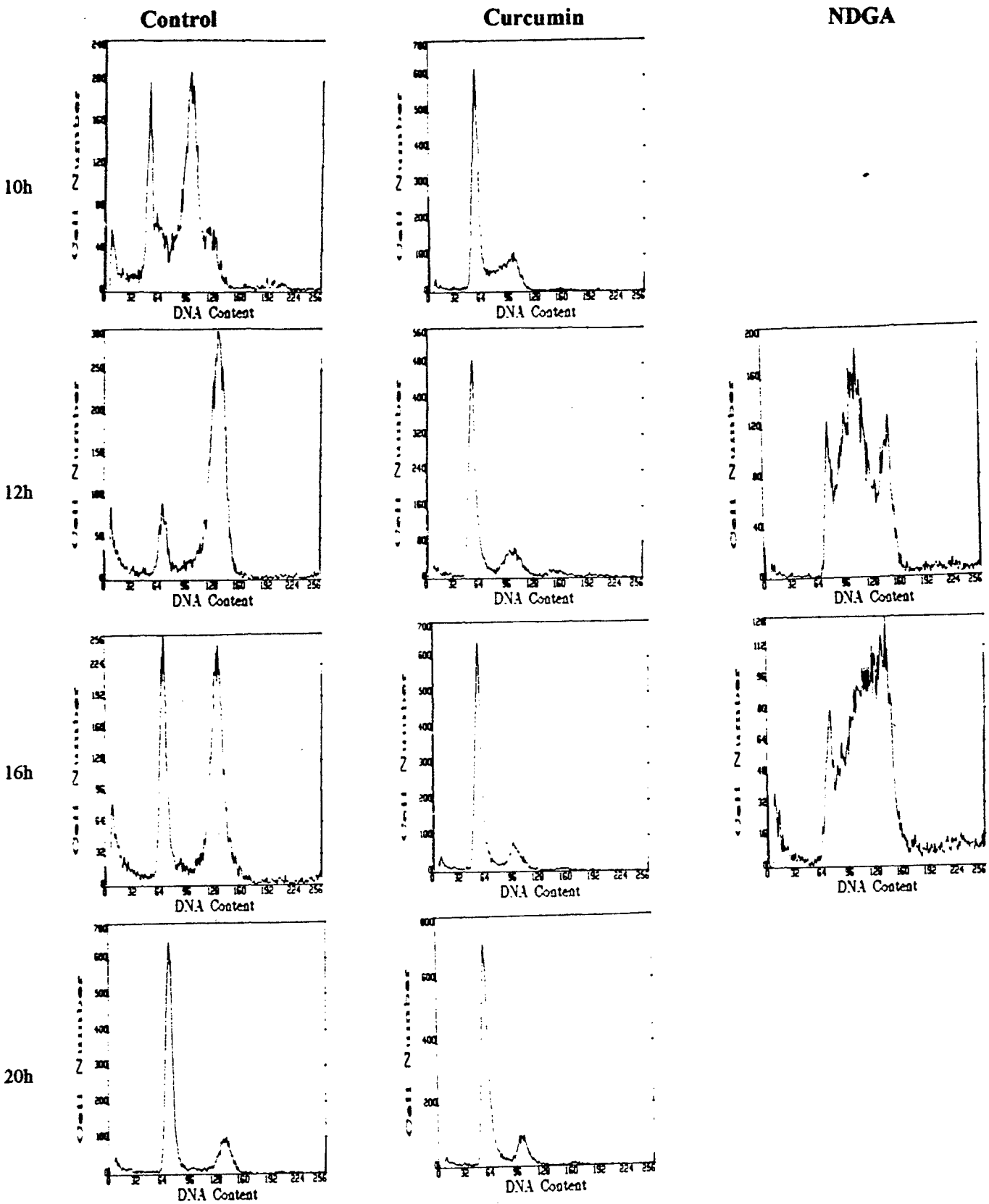
DNA CONTENT - PROPIDIUM IODIDE STAINING

Treatment of synchronized MCF-7 (ADR) cells with Lipoxygenase Inhibitor



DNA Content of Propidium Iodide Stained (FL2-A) MCF-7 cells treated with 50μM Curcumin or 50μM NDGA

Figure 5. Continued



DNA Content of Propidium Iodide Stained (FL2-A) MCF-7 cells treated with 50μM Curcumin or 50μM NDGA

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Figure 11
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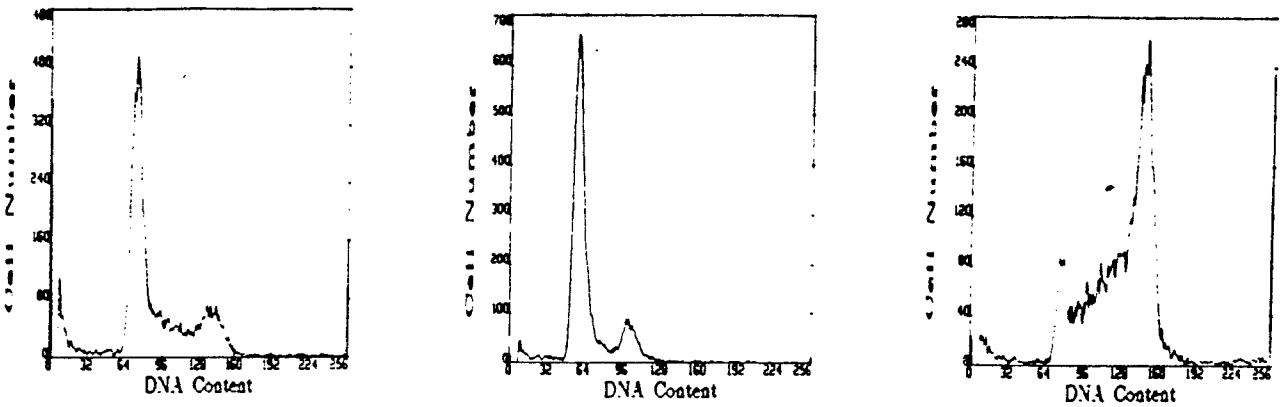
Control **Curcumin** **NDGA**

24h



DNA Content of Propidium Iodide Stained (FL2-A) MCF-7 cells treated with 50μM Curcumin or 50μM NDGA

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Figure 12 A
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Human Bone Marrow stroma colony assay

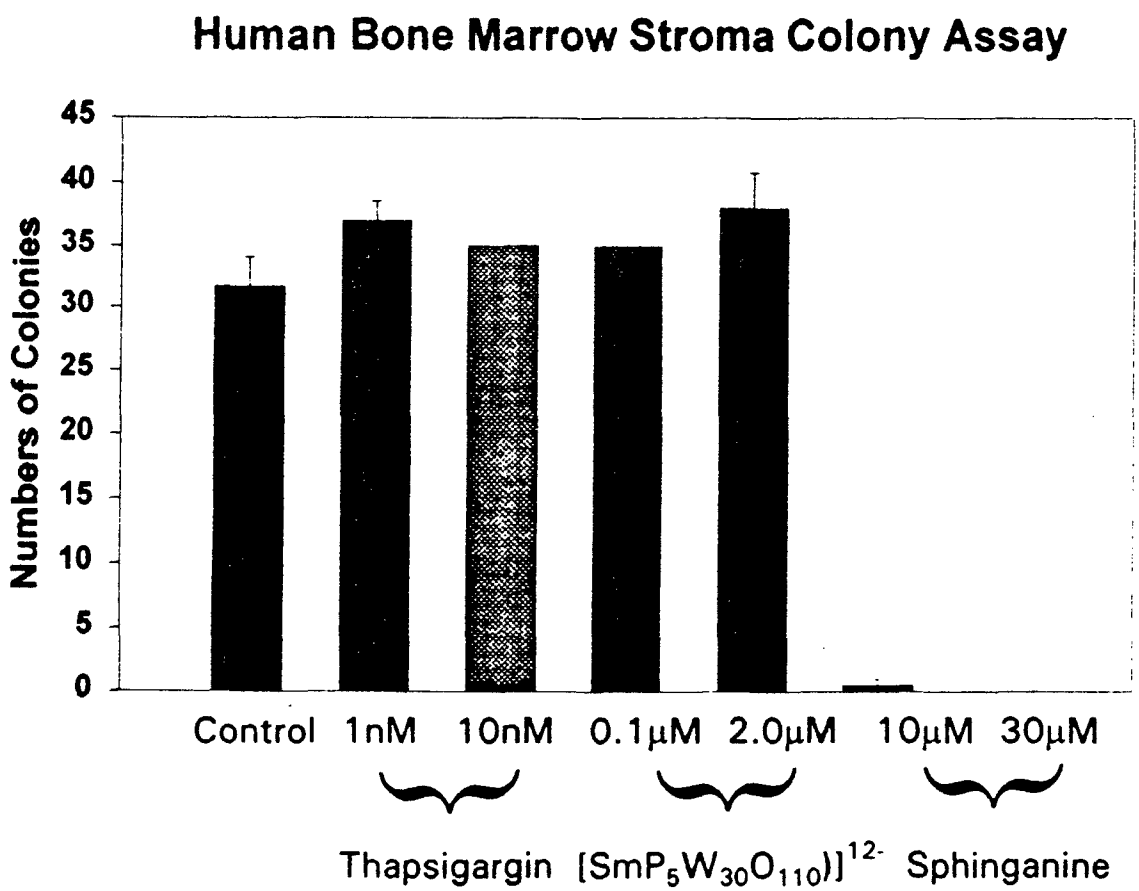

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Figure 12 B
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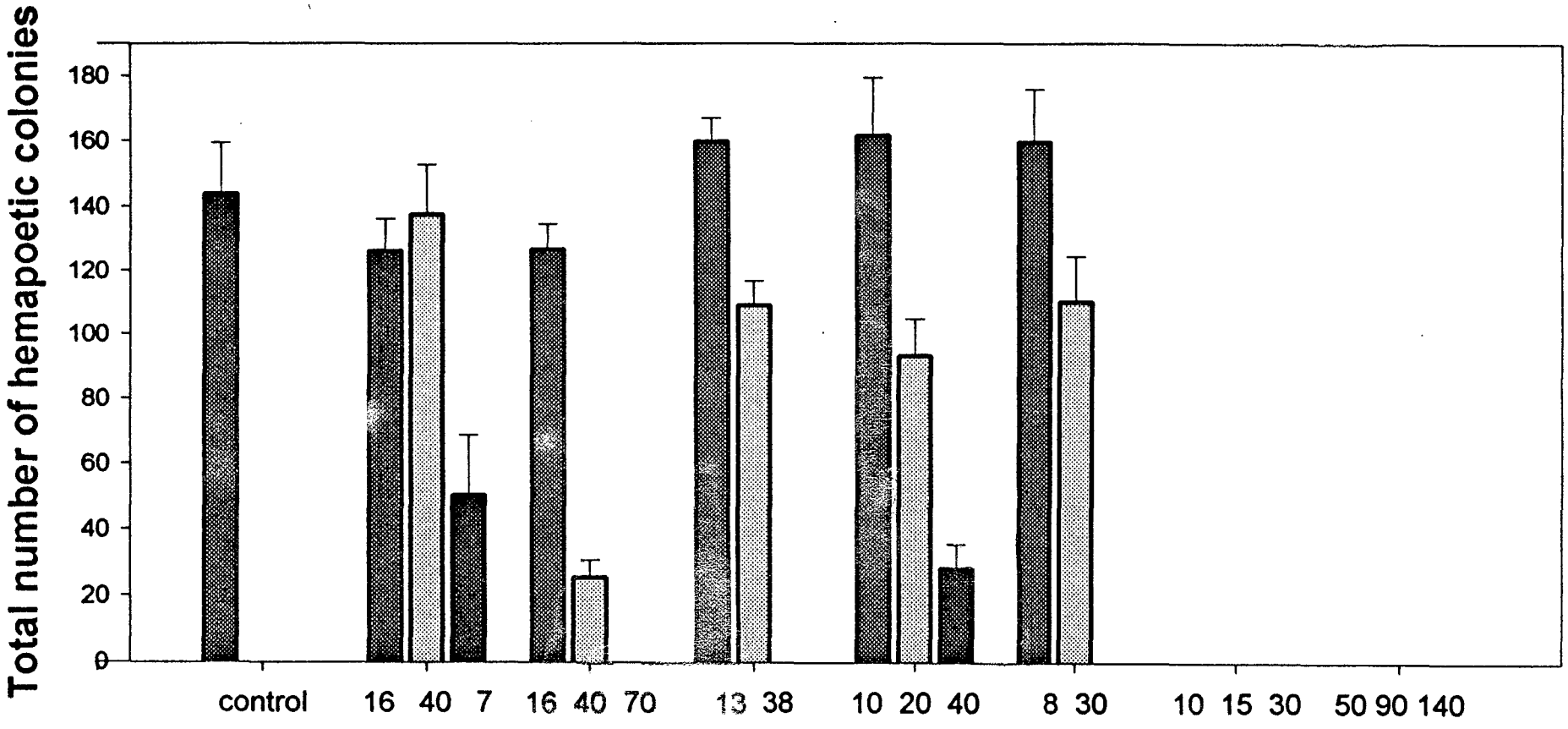

The treatment of human bone marrow cells with the drugs at different concentration (μM)

Received 1-28 2000 ack

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 7 Jan 00

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCA, 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for Grants listed below. Request the limited distribution statements for enumerated Accession Document Numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service:

GRANT	ACCESSION DOCUMENT NUMBER
AIBS NUMBER 2691	ADB206345, ADB226405
~~DAMD17-94-J-4057~~	~~ADB234542~~ - cited in ltr of 4 Jan 2000

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by email at Judy.Pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Phylis Rinehart
PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Completed 1-31-00 ack